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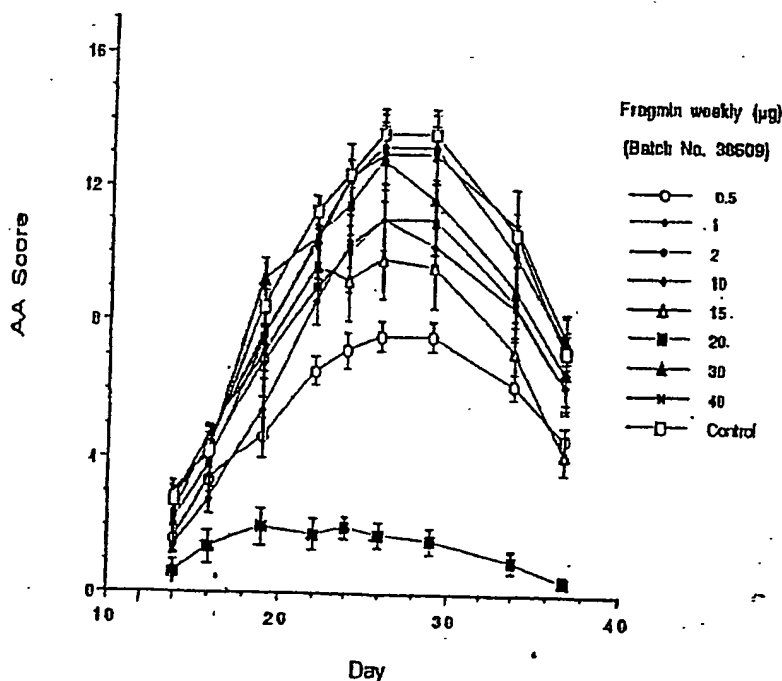
(57) Abstract

The present invention relates to pharmaceutical compositions for the prevention and/or treatment of pathological processes involving the induction of TNF-α secretion comprising a pharmaceutically acceptable carrier and a low molecular weight heparin (LMWH). In the pharmaceutical compositions of the present invention, the LMWH is present in a low effective dose and is administered at intervals of about 5-8 days. Furthermore, the LMWH is capable of inhibiting *in vitro* TNF-α secretion by resting T cells and/or macrophages in response to T cell-specific antigens, mitogens, macrophage activators, disrupted extracellular matrix (dECM), laminin, fibronectin, and the like.

-1-

COMPOSITIONS FOR THE PREVENTION AND/OR
TREATMENT OF PATHOLOGICAL PROCESSES

1. Field of the Invention

The present invention relates to
pharmaceutical compositions for the prevention and/or
treatment of pathological processes involving
induction of Tumor Necrosis Factor alpha (TNF-α)
secretion, which comprise an effective low dosage of a
low molecular weight heparin (LMWH) to be administered
at intervals of between five to eight days.

The LMWHs to be used according to the
invention have an average molecular weight of from
about 3,000 to about 6,000 and inhibit TNF-α secretion
by resting T cells and/or macrophages in vitro in
response to activation by T cell-specific antigens,
T cell mitogens, macrophage activators, disrupted
extracellular matrix (dECM), fibronectin, laminin or
other ECM components.

2. Background of the Invention

TNF-α, a cytokine produced by monocytes
(macrophages) and T lymphocytes, is a key element in
the cascade of factors that produce the inflammatory
response and has many pleiotropic effects as a major
orchestrator of disease states (B. Beutler and A.
Cerami, Ann. Rev. Immunol. (1989) 7:625-655).

The biologic effects of TNF-α depend on its
concentration and site of production: at low
concentrations, TNF-α may produce desirable
homeostatic and defense functions, but at high
concentrations, systemically or in certain tissues,
TNF-α can synergize with other cytokines, notably
interleukin-1 (IL-1) to aggravate many inflammatory
responses.

The following activities have been shown to
be induced by TNF-α (together with IL-1); fever,

slow-wave sleep, hemodynamic shock, increased
production of acute phase proteins, decreased
production of albumin, activation of vascular
endothelial cells, increased expression of major
5   histocompatibility complex (MHC) molecules, decreased
lipoprotein lipase, decreased cytochrome P450,
decreased plasma zinc and iron, fibroblast
proliferation, increased synovial cell collagenase,
increased cyclo-oxygenase activity, activation of T
10   cells and B cells, and induction of secretion of the
cytokines, TNF-α itself, IL-1 and IL-6.

How TNF-α exerts its effects is not known in
detail, but many of the effects are thought to be
related to the ability of TNF-α to stimulate cells to
15   produce prostaglandins and leukotrienes from
arachidonic acid of the cell membrane.

TNF-α, as a result of its pleiotropic
effects, has been implicated in a variety of
pathologic states in many different organs of the
20   body. In blood vessels, TNF-α promotes hemorrhagic
shock, down regulates endothelial cell thrombomodulin
and enhances a procoagulant activity. It causes the
adhesion of white blood cells and probably of
platelets to the walls of blood vessels, and so, may
25   promote processes leading to atherosclerosis, as well
as to vasculitis.

TNF-α activates blood cells and causes the
adhesion of neutrophils, eosinophils, monocytes/
macrophages and T and B lymphocytes. By inducing IL-6
30   and IL-8, TNF-α augments the chemotaxis of
inflammatory cells and their penetration into tissues.
Thus, TNF-α has a role in the tissue damage of
autoimmune diseases, allergies and graft rejection.

TNF-α has also been called cachectin because
35   it modulates the metabolic activities of adipocytes
and contributes to the wasting and cachexia
accompanying cancer, chronic infections, chronic heart

failure, and chronic inflammation. TNF-α may also
have a role in anorexia nervosa by inhibiting appetite
while enhancing wasting of fatty tissue.

5   TNF-α has metabolic effects on skeletal and
cardiac muscle. It has also marked effects on the
liver: it depresses albumin and cytochrome P450
metabolism and increases production of fibrinogen,
α1-acid glycoprotein and other acute phase proteins.
It can also cause necrosis of the bowel.

10   In the central nervous system, TNF-α crosses
the blood-brain barrier and induces fever, increased
sleep and anorexia. Increased TNF-α concentration is
associated with multiple sclerosis. It further causes
adrenal hemorrhage and affects production of steroid

15 hormones, enhances collagenase and PGE-2 in the skin,
and causes the breakdown of bone and cartilage by
activating osteoclasts.

             In short, TNF-α is involved in the
pathogenesis of many undesirable inflammatory

20 conditions in autoimmune diseases, graft rejection,
vasculitis and atherosclerosis. It may have roles in
heart failure, in the response to cancer and in
anorexia nervosa. For these reasons, ways have been
sought to regulate the secretion of TNF-α as a means

25 to control a variety of diseases.

             The prime function of the immune system is
to protect the individual against infection by foreign
invaders such as microorganisms. It may, however,
also attack the individual's own tissues leading to

30 pathologic states known as autoimmune diseases. The
aggressive reactions of an individual's immune system
against tissues from other individuals are the reasons
behind the unwanted rejections of transplanted organs.
Hyper-reactivity of the system against foreign

35 substances causes allergy giving symptoms like asthma,
rhinitis and eczema.

  The cells mastering these reactions are the
lymphocytes, primarily the activated T lymphocytes,
and the pathologic inflammatory response they direct
depends on their ability to traffic through blood

5 vessel walls to and from their target tissue. Thus,
reducing the ability of lymphocytes to adhere to and
penetrate through the walls of blood vessels may
prevent autoimmune attack, graft rejection and
allergy. This would represent a new therapeutic

10 principle likely to result in better efficacy and
reduced adverse reactions compared to the therapies
used today.

  Atherosclerosis and vasculitis are chronic
and acute examples of pathological vessel

15 inflammation. Atherosclerosis involves thickening and
rigidity of the intima of the arteries leading to
coronary diseases, myocardial infarction, cerebral
infarction and peripheral vascular diseases, and
represents a major cause of morbidity and mortality in

20 the Western world. Pathologically, atherosclerosis
develops slowly and chronically as a lesion caused by
fatty and calcareous deposits. The proliferation of
fibrous tissues leads ultimately to an acute condition
producing sudden occlusion of the lumen of the blood

25 vessel.

  TNF-α has been shown to facilitate and
augment human immunodeficiency virus (HIV) replication
in vitro (Matsuyama, T. et al., _J. Virol._ (1989)
_63(6)_:2504-2509; Michihiko, S. et al., _Lancet_ (1989)

30 _1(8648)_:1206-1207) and to stimulate HIV-1 gene
expression, thus, probably triggering the development
of clinical AIDS in individuals latently infected with
HIV-1 (Okamoto, T. et al., _AIDS Res. Hum. Retroviruses_
(1989) _5(2)_:131-138).

35 Heparin is a glycosaminoglycan, a
polyanionic sulfated polysaccharide, which is used
clinically to prevent blood clotting as an

antithrombotic agent. In animal, models heparin has
been shown to reduce the ability of autoimmune T
lymphocytes to reach their target organ (Lider, O. et
al., Eur. J. Immunol. (1990) 20:493-499). Heparin was
5   also shown to suppress experimental autoimmune
diseases in rats and to prolong the allograft survival
in a model of skin transplantation in mice, when used
in low doses (5 µg for mice and 20 µg for rats)
injected once a day (Lider, O. et al., J. Clin.
10 Invest. (1989) 83:752-756).

The mechanisms behind the observed effects
are thought to involve inhibition of release by T
lymphocytes of enzyme(s) necessary for penetration of
the vessel wall, primarily the enzyme heparanase that
15 specifically attacks the glycosaminoglycan moiety of
the sub-endothelial extracellular matrix (ECM) that
lines blood vessels (Naparstek, Y. et al., Nature
(1984) 310:241-243). Expression of the heparanase
enzyme is associated with the ability of autoimmune T
20 lymphocytes to penetrate blood vessel walls and to
attack the brain in the model disease experimental
autoimmune encephalomyelitis (EAE).

European Patent Application EP 0114589
(Folkman et al.) describes a composition for
25 inhibition of angiogenesis in mammals in which the
active agents consist essentially of (1) heparin or a
heparin fragment which is a hexasaccharide or larger
and (2) cortisone or hydrocortisone or the 11-α isomer
of hydrocortisone. According to the disclosure,
30 heparin by itself or cortisone by itself are
ineffective; only the combination of both gives the
desired effects. Although there is no proof in the
literature that there is a connection between
angiogenesis and autoimmune diseases, the description
35 on page 5 of the patent application connects
angiogenesis with psoriasis and with arthritis,

indicating the use of high doses of 25,000 units to
47,000 units of heparin per day.

Horvath, J.E. et al., Aust. N.Z.J. Med.
(1975) 5(6):537-539 describe the effect of
5   subanticoagulant doses of subcutaneous heparin on
early renal allograft function. The daily dosage is
high (5000 U) and the conclusion of the study is that
heparin in subanticoagulant doses has no effect on
early graft function or graft survival and that it may
10 be associated with increased hemorrhagic
complications.

Toivanen, M.L. et al., Meth. and Find. Exp.
Clin. Pharmacol. (1982) 4(6):359-363, examined the
effect of heparin in high dosage (1000 U/rat) in the
15 inhibition of adjuvant arthritis in rats and found
that heparin enhanced the severity of the rat adjuvant
arthritis.

PCT Patent: Application PCT/AU88/00017
published under No. WO88/05301 (Parish et al.)
20 describes sulphated polysaccharides that block or
inhibit endoglycosylase activity, such as heparanase
activity, for use as antimetastatic and
anti-inflammatory agents. Heparin and heparin
derivatives, such as periodate oxidized, reduced
25 heparins, that had negligible anticoagulant activity,
were shown to have antimetastatic and
anti-inflammatory activity when used in dosages
within, the range of 1.6-6.6 mg per rat daily,
administered by constant infusion (corresponding to
30 75-308 mg daily for an adult human patient).

3.   Summary of the Invention

The present invention is related to
pharmaceutical compositions for the prevention and/or
35 treatment of pathological processes involving the
induction of TNF-α secretion comprising a
pharmaceutically acceptable carrier and a low

molecular weight heparin (LMWH) present in a low
effective dose for administration at intervals of
about 5-8 days and which LMWH is capable of inhibiting
in vitro TNF-α secretion by resting T cells and/or
5 macrophages in response to T cell-specific antigens,
mitogens, macrophage activators, disrupted
extracellular matrix (dECM), laminin, fibronectin, or
other ECM components.

       In a particular embodiment of the present
10 invention the LMWH of the pharmaceutical composition
has an average molecular weight of from about 3,000 to
about 6,000 and, furthermore, may be administered
every fifth or seventh day.

       It is also an objective of the present
15 invention to provide pharmaceutical compositions to be
administered at intervals of about 5-8 days for the
prevention and/or treatment of pathological processes
involving the induction of TNF-α secretion comprising
a pharmaceutically acceptable carrier and a low
20 molecular weight heparin (LMWH) present in a low
effective dose and which LMWH is capable of inhibiting
experimental delayed type hypersensitivity (DTH)
reactions to an applied antigen as evidenced by a
reduction in the induration observed after the
25 application of the antigen to the skin five to seven
days after the administration of the pharmaceutical
composition relative to the induration observed after
the application of the antigen to the skin in the
absence of or after recovery from the administration
30 of the pharmaceutical composition. Examples of the
applied antigen include, but are not limited to,
tetanus, myelin basic protein, purified protein
derivative, oxazolone, and the like.

       Furthermore, it is an objective of the
35 present invention to provide pharmaceutical
compositions that may be administered in any manner as
dictated by the particular application at hand

including, but not limited to, enteral administration
(including oral) or parenteral administration
(including topical or inhalation with the aid of
aerosols). In preferred embodiments, the
5 pharmaceutical compositions of the present invention
are administered subcutaneously or intravenously.

Thus, the present invention is useful, for
example, in delaying or preventing allograft rejection
and treating or preventing a variety of pathological
10 processes such as those related to autoimmune
diseases, allergy, inflammatory diseases (in
particular, inflammatory bowel disease), or acquired
immunodeficiency syndrome (AIDS). The present
invention also finds utility in the treatment of
15 diabetes type I, periodontal disease, uveitis,
rheumatic diseases (in particular, rheumatoid
arthritis), atherosclerosis, vasculitis, or multiple
sclerosis.

The pharmaceutical compositions of the
20 present invention contain low effective doses of the
prescribed LMWH and typically contain a single low
dose unit of less than 5 mg of LMWH, preferably from
about 0.3 to about 3 mg, and most preferably contain a
single low dose unit of from 1 to 1.5 mg.

25   The present invention also contemplates
broadly a method of using a low molecular weight
heparin (LMWH) which is capable of inhibiting in vitro
TNF-α secretion by resting T cells and/or macrophages
in response to T cell-specific antigens, mitogens,
30 macrophage activators, disrupted extracellular matrix
(dECM), laminin, fibronectin, or other ECM components
for the preparation of a pharmaceutical composition to
be administered at intervals of about 5-8 days for the
prevention and/or treatment of pathological processes
35 involving induction of TNF-α secretion which method
comprises combining a low effective dose of the LMWH
with a pharmaceutically acceptable carrier.

- 9 -

     A still further object of the present invention is to provide a method of treating a subject suffering from a pathological process involving induction of TNF-α secretion comprising administering
5 to such subject a pharmaceutical composition, as described above, at intervals of about 5-8 days.
    Further objects of the present invention will become apparent to those skilled in the art upon further review of the following disclosure, including
10 the detailed descriptions of specific embodiments of the invention.

     4. Detailed Description of the Invention
     According to the present invention, it was
15 found that treatment with low molecular weight heparins (LMWHs) inhibited the ability of T cells and macrophages to secrete TNF-α. The functional expression of this effect can be seen in the inhibition in mice and humans of the delayed type
20 hypersensitivity (DTH) reaction, an inflammatory reaction triggered by cells involving macrophages and other inflammatory cells. Treatment with LMWHs at doses affecting TNF-α production also was able to inhibit a model of autoimmune arthritis called
25 adjuvant arthritis (AA). LMWH treatment also prolonged the survival of allogeneic heart transplants in rats and abrogated insulin dependent diabetes mellitus (IDDM) in NOD mice. Moreover, treatment with LMWHs prevented the induction of TNF-α production by T
30 cells and macrophages in response to the stimulus of damaged subendothelial extracellular matrix (ECM). Since TNF-α at the site of vascular injury has probably a role in the process of atherosclerosis, inhibition of TNF-α at the site of damaged
35 subendothelial ECM will ameliorate the pathogenic process of atherosclerosis. A most surprising aspect of treatment with LMWHs is that such treatment is most

effective when administered at low doses at weekly
intervals. High doses of LMWHs or doses of LMWHs
given daily are not effective in inhibiting TNF-α
secretion or immune reactions.

5   Low molecular weight heparins, produced by
fractionation or controlled depolymerization of
heparins, show improved antithrombotic performance but
also different pharmacokinetic properties as compared
to heparin: the half-life is doubled and the
10   bioavailability is higher with respect to their,
anticoagulant effect after subcutaneous injection
(Bratt, G. et al. Thrombosis and Haemostasis (1985)
53:208; Bone, B. et al., Thrombosis Research (1987)
46:845).

15   According to the present invention it has
now been found that LMWHs administered at
subanticoagulant doses at several day intervals are
effective in the prevention and/or treatment of
pathological processes involving induction of TNF-α.

20   The LMWHs to be used according to the
invention are derived from LMWHs with an average
molecular weight of 3000-6000, such as, for example
the LMWHs disclosed in European Patent EP 0014184.
Some LMWHs are commercially available under different
25   trade names, e.g., Fragmin®, Fraxiparin®,
Fraxiparine®, Lovenox®/Clexane®.

          LMWHs can be produced in several different
ways: enrichment by fractionation by ethanol and/or
molecular sieving, e.g., gel filtration or membrane
30   filtration of the LMWH present in standard heparin and
controlled chemical (by nitrous acid, β-elimination or
periodate oxidation) or enzymatic (by heparinases)
depolymerization. The conditions for depolymerization
can be carefully controlled to yield products of
35   desired molecular weights. Nitrous acid
depolymerization is commonly used. Also employed is
depolymerization of the benzylic ester of heparin by

- 11 -

β-elimination, which yields the same type of fragments
as enzymatic depolymerization using heparinases. LMWH
with low anticoagulant activity and retaining basic
chemical structure can be prepared by depolymerization
5   using periodate oxidation or by removing the
antithrombin-binding fraction of LMWH, prepared by
other methods, using immobilized antithrombin for
adsorption.

Fragmin® is a low molecular weight heparin
10   with average molecular weight within the range of
4000-6000 dalton, produced by controlled nitrous acid
depolymerization of sodium heparin from porcine
intestinal mucosa. It is manufactured by Kabi
Pharmacia, Sweden, under the name Fragmin®, for use as
15   an antithrombotic agent as saline solutions for
injection in single dose syringes of 2500 IU/0.2 ml
and 5000 IU/0.2 ml, corresponding to about 16 mg and
32 mg, respectively.

Fraxiparin®, and Fraxiparine® are LMWHs with
20   average molecular weight of approximately 4500 dalton,
produced by fractionation or controlled nitrous acid
depolymerization, respectively, of calcium heparin
from porcine intestinal mucosa. It is manufactured by
Sanofi (Choay Laboratories) for use as an antithrombo-
25   tic agent in single doses comprising ca. 36 mg,
corresponding to 3075 IU/0.3 ml of water.

Lovenox® (Enoxaparin/e), a LMWH fragment
produced by depolymerization of sodium heparin from
porcine intestinal mucosa, using β-elimination, is
30   manufactured by Pharmuka SF, France and distributed by
Rhone-Poulenc under the names Clexane® and Lovenox®
for use as antithrombotic agent in single dose
syringes comprising 20 mg/0.2 ml and 40 mg/0.4 ml of
water.
35   As shown in the present application, the
novel properties of LMWHs described are a common
feature of all, LMWHs regardless of the manufacturing

process, the structural differences (created by depolymerization or those dependent on variation in the heparin used as raw material) or the anticoagulant activity, provided the LMWH employed is capable of

5   inhibiting TNF-α secretion in vitro by resting T cells and/or macrophages in response to activation by contact with specific antigens, mitogens, disrupted ECM or its protein components, such as fibronectin or laminin.

10   Another test useful to identify the LMWHs that are effective for the purpose of the present invention is the inhibition of experimental delayed type hypersensitivity (DTH) skin reactions, a T lymphocyte dependent reaction, to a variety of

15   antigens (for example, tetanus antigen, myelin basic protein (MBP), purified protein derivative (PPD), and oxazolone). The LMWHs also inhibit T cell adhesion to ECM and its protein components.

         The LMWHs effective according to the

20   invention are incorporated into pharmaceutical compositions, for example, as water solutions, possibly comprising sodium chloride, stabilizers and other suitable non-active ingredients. The preferable way of administration is by injection, subcutaneous or

25   intravenous, but any other suitable mode of administration is encompassed by the invention.

         According to the invention, the LMWH is to be administered at intervals of five to eight days, preferably once a week.

30   The disorders that can be prevented or treated according to the invention are all disorders linked in pathological processes involving induction of TNF-α secretion, including atherosclerosis and vasculitis and pathological processes related thereto;

35   autoimmune diseases, e.g., rheumatoid arthritis, diabetes type I (IDDM); allergy; graft rejection; acute and chronic inflammatory diseases, e.g. uveitis,

- 13 -

bowel inflammation; anorexia nervosa; hemorrhagic
shock caused by septicemia, and opportunistic
infections in AIDS - immunocompromised individuals.
In AIDS, the LMWHs will suppress replication of HIV
5 and/or enhance resistance against the opportunistic
infections, thereby preventing the development of
AIDS-related complex (ARC).

    It was observed according to the present
invention that LMWHs can have a narrow dose range of
10 effectiveness, and that different preparations of
LMWHs can show different optimal biological effects.
Therefore, it was necessary to establish a Unit Dose
for LMWH preparation to be used according to the
invention, said unit dose being based on a bioassay of
15 inhibition of TNF-α secretion by mouse spleen cells
from mice treated with LMWH or by inhibition of DTH
reactivity by t... LMWH. It was found that the ability
of a particular dose of LMWH to inhibit TNF-α
secretion is positively correlated with its ability to
20 inhibit the delayed type hypersensitivity (DTH)
reaction. This, cell-mediated inflammatory reaction
in vivo is important because it is an expression of
the processes involved in autoimmune diseases, graft
rejection, some types of blood vessel inflammation and
25 allergy.

    It was thus established according to the
invention that the lowest dose of LMWH per kg causing
inhibition of TNF-α, production or inhibition of DTH
reactivity by at least 50% is considered to constitute
30 12 mouse inhibitory units per kg (12 u/kg). Because
ᵣ⁻ the differences in surface area and metabolism
L-tween mice and humans, humans should be treated with
a lower dose of LMWH, and 12 u/kg in mouse is
established to correspond to 1 u/kg in humans. For
35 example, the dose of Fragmin® batch 38609 effective in
inhibiting both TNF-α secretion and DTH reactivity is
5 μg per mouse administered weekly. Since each mouse

- 14 -

weighs about 25 g, the dose of Fragmin® 38609
equivalent to 12 u/kg is 200 μg/kg of mice. The dose
of 1 u/kg suitable for humans is therefore 200 μg/kg:
12 = 16.67 μg/kg. A human weighing about 70 kg would
5   then be treated by a dose of about 1.2 mg given in a
single dose subcutaneously once every 7 days. Since
individual humans vary biologically, the optimal dose
may be different from about 1.2 mg and will lie below
5 mg, particularly within the range of 0.3 to 3 mg.

10   The dose of LMWH that should be effective in
rats can be derived from the fact that the dose of
LMWH per kg of rats is one-half the dose per kg of
mice, i.e. 6 u/kg. For example, if 12 u of Fragmin®
batch 38609 is 200 μg/kg, then the 6 u dose suitable
15   for rats should be 100 μg/kg or 20 μg per 200 g rat,
administered once a week.

The invention will now be illustrated by the
following non-limiting examples.

20   5.   Examples
5.1.   Example 1: Bioassay of Inhibition
                     of TNF-α Secretion

Supernatants of spleen cells cultured in the
presence or absence of LMWH, or spleen cells obtained
from mice treated or untreated with LMWH in vivo are
25   analyzed for their ability to secrete TNF-α. The
TNF-α bioassay is based on the cytotoxic effect of
TNF-α on cycloheximide (CHI)-sensitized cells and its
quantitation by the neutral red uptake assay as
described by Wallach D., J. Immunology (1984)
30   132:2464-2469. Briefly, the killing of CHI-sensitized
HeLa cells by TNF-α present in the supernatants of the
cells is measured, the concentration of TNF-α in said
supernatants being determined by comparison to
titration curves of TNF-α exogenously added. Cell
35   viability is determined by incubation with neutral red
for two hours, washing away excess dye, extracting the

- 15 -

neutral red that was taken up by the cells with
Sorenson's citrate buffer-ethanol mixture, and
quantitating it colorimetrically at 570 nm with a
Microelisa Autoreader.

5   Cells from mice treated with LMWH are
obtained as follows: female mice of the BALB/c strain
(25 grams, 2 months old), at least 5 mice per group,
are injected subcutaneously with various doses of
LMWH, usually in the range of 0.5 to 20 μg per mouse.
10 Five days later the mice are killed by cervical
dislocation, the spleens are removed and suspensions
of spleen cells, depleted of red blood cells, are
assayed for the production of TNF-α in response to
induction by dECM, Concanavalin A (Con A) or
15 lipopolysaccharide (LPS).

        5.2. Example 2: Bioassay of Inhibition
              of DTH Reactivity

        Groups of mice are sensitized on the shaved
20 abdominal skin with 100 μl of 3% oxazolone (OX) in
acetone/olive oil (4:1, v:v). DTH sensitivity is
elicited 5 days later as follows: mice are challenged
with 20 μl of 0.5% OX (10 μl to each side of the ear)
in acetone/olive oil. A constant area of the ear is
25 measured immediately before challenge and 24 and 48 h
later with a Mitutoyo engineer's micrometer. The
individual measuring ear swelling is blinded to the
identity of the groups of mice. The increment (Δ) of
ear swelling is expressed as the mean in units of 10^{-2}
30 mm or 10^{-4} inch ($\pm$SE) depending on the micrometer that
is used. Percent inhibition is calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{Positive control} - \text{experimental group}}{\text{Positive control} - \text{negative control}} \times 100$$

        Mice are treated with LMWH as in Example 1,
35 injected the day before primary sensitization to OX.
On the fifth day after sensitization to OX, the mice

- 16 -

are challenged to induce a DTH reaction, as described
above.

The positive control is the DTH reaction
elicited in immunized mice in the absence of treatment
5   with LMWH. The negative control is the background
swelling produced by the antigen in immunized mice.

5.3.   Example 3: Induction of TNF-α Secretion
by T Cells and Macrophages In Vitro

Microtiter plates were prepared as follows:
10   fibronectin (FN) or laminin (LN) (Sigma) were added to
flat bottom 96-well plates (Costar) at a concentration
of 1 µg/50 µl PBS per well and removed after 16 h.
Remaining binding sites were blocked with BSA/PBS (10
mg/ml) which was added to the wells for 2 h and washed
15   out. ECM-coated wells were prepared as follows:
bovine corneal endothelial cells were cultured in flat
bottom 96-well plates. The confluent layers of
endothelial cells were dissolved and the ECM was left
intact free of cellular debris (Gospodarowicz, D. et
20   al. J. Biol. Chem. (1978) 253:3736). Disrupted ECM
(dECM) was prepared by gently scratching the ECM three
times with a 27G syringe needle and the exposed sites
were subsequently coated with BSA/PBS. Resting cloned
rat CD4⁺ T cells, designated K1, which recognize
25   myelin basic protein (MBP), were propagated and
maintained in culture and were added to the wells, 10⁵
cells per well with or without 3 x 10⁵ syngeneic
splenic macrophages, in 100 µl per well RPMI 1640
30   (Gibco) supplemented with 1% BSA and antibiotics. The
splenic macrophages were purified by removing the T
and B cells using specific monoclonal antibodies
(mAb). Where indicated, anti-murine TNF-α mAb
(Genzyme, diluted 1/300) were added to the wells. MBP
35   (100 µg/ml), Con A (2.5 µg/ml), LPS (1 µg/ml) were
added to the wells where indicated. The plates were
incubated at 37 °C in a humidified incubator for 3 h.

Subsequently, the contents of the wells (4 wells per
experimental group) were collected, centrifuged, and
the media were assayed for TNF-α secretion as in
Example 1: supernatants of cultured macrophages and
5 lymphocytes were added to cultures of HeLa cells,
which are sensitive to killing by TNF-α, and death of
these cells in the presence of the test media was
calibrated in comparison to titration curves of
exogenous added TNF-α. Cell death is examined by the
10 release of neutral red dye from the preincubated HeLa
cells. The results shown here represent data obtained
from a total of six experiments that produced
essentially similar results.

Table 1 shows that T cells and macrophages
15 cultured together can be induced to secrete TNF-α by
contact with specific antigen MBP (group 4), the
mitogen Con A (group 6) or LPS (group 8). However, in
the absence of antigenic or mitogenic stimulus, TNF-α
was also induced to be secreted by disrupted
20 extracellular matrix (dECM; group 10) or by the ECM
components fibronectin (FN; group 12) or laminin (LN
group 14). Intact ECM was a weak inducer of TNF-α
(group 16).

25 **Table 1.** TNF-α secretion by T cells and macrophages
is induced by specific antigen MBP, Con A, LPS or dECM
components.

Group	TNF-α inducer	K1 cells cultured together with (yes) or without (no) macrophages	Secreted TNF-α (pg/ml)
1	none	no	50
2		yes	65
3	MBP antigen	no	30
4		yes	950
5	Con A	no	120
6		yes	1300

- 18 -

	7	LPS	no	50
	8		yes	1500
	9	dECM	no	30
5	10		yes	900
	11	FN	no	20
	12		yes	650
	13	LN	no	50
	14		yes	500
10	15	ECM	no	30
	16		yes	120

5.4.   Example 4:   Regulation of TNF-α Secretion by LMWHs

T cell and accessory cell cultures were prepared as described in Example 3. LMWH was added to the wells at the beginning of the cell culture. The levels of TNF-α were examined after 3 h of incubation.

Table 2 shows that the presence of LMWH (Fragmin® batch 38609) in vitro inhibited the induction of TNF-α secretion induced by specific antigen (MBP; group 4), mitogens (Con A and LPS; groups 6 and 8), dECM or ECM components (groups 10, 12 and 14). Since TNF-α secretion induced by dECM is likely to be involved in atherosclerosis, inhibition of TNF-α by LMWH will be beneficial in atherosclerosis.

Table 2. Induction of TNF-α secretion induced in vitro is inhibited by LMWH (Fragmin® batch 38609).

Group	TNF-α Inducer	LMWH (1 μg/ml)	Secretion of TNF-α by cultures of T cells and macrophages (pg/ml)
1	none	none	65
2		yes	30

- 19 -

3	MPB antigen	none		950
4		yes		60
5	Con A	none		1300
6		yes		80
7	LPS	none		1500
8		yes		80
9	dECM	none		900
10		yes		90
11	FN	none		650
12		yes		90
13	LN	none		500
14		none		70

5.5. Example 5

To examine the effect of LMWH administered
to mice in vivo on the secretion of TNF-α by spleen
cells in vitro, the following experiment was
conducted. BALB/c mice, 5 per group, were treated
with various doses of LMWH (Fragmin® batch 38609)
diluted in saline, injected subcutaneously. After one
week, the animals were killed and their spleen cells,
devoid of red blood cells, were examined for their
ability to secrete TNF-α in response to control wells
without dECM (A) or to wells coated with dECM (B).
Measuring the levels of TNF-α secretion was done as
described in Example 1. Table 3 shows the results
which indicate that an injection of 5 μg of LMWH given
once, 7 days earlier, inhibited TNF-α secretion
induced by dECM. Higher or lower doses of LMWH were
less effective. Thus, an optimal dose of LMWH
administered in vivo a week earlier was effective.

20

Table 3. Ex vivo inhibition of T cell mediated TNF-α secretion in response to damaged ECM.

LMWH treatment of BALB/c mice (weekly)	In vitro TNF-α secretion (pg/ml) by spleen cells cultured on:		
	A. None	B. Damaged ECM	(% Inhibition)
1 None	30	400	
2 0.5 μg	50	380	(5)
3 1 μg	25	90	(78)
4 5 μg	25	60	(85)
5 10 μg	30	140	(65)
6 20 μg	40	320	(20)

Table 4 shows that a 5 μg dose in vivo of
the LMWH Fragmin® batch 38609 was also effective in
inhibiting TNF-α secretion induced by LPS. BALB/c (4
mice per experimental group) mice were treated with
the indicated amounts of LMWH diluted in saline and
injected subcutaneously. After one week, the mice
were injected intraperitoneally with 10 mg LPS, killed
4 hours later and their spleen cells, devoid of red
blood cells, were subsequently cultured in dECM coated
wells for 3 hours in a humidified incubator. The
levels of TNF-α secreted in response to the dECM was
measured in the supernatants of the cultures. The
results are given in Table 4.

Table 4. Treatment of mice with LMWH inhibits LPS
mediated secretion of TNF-α by macrophages.

LMWH treatment of mice (μg)	In vitro TNF-α secretion by macrophages (pg/ml) in response to LPS	% Inhibition
0	690	--
0.1	500	28
1	350	50

21

5	120	82
20	550	20

5   5.6. Underline Example 6

To examine the effect of different LMWHs on
the inhibition of TNF-α secretion and on DTH
responses, mice were treated with the indicated LMWH
administered subcutaneously in different
10 concentrations. After one week, some of the mice were
killed and the induction of TNF-α secretion in
response to Con A activation in vitro was measured.
The remaining mice were examined for their ability to
respond to the antigen oxazolone. The results are
15 expressed in Table 5 as percent inhibition compared to
the responses of the LMWH untreated mice.

Two conclusions can be made by inspecting
the results shown in Table 5:

1. Different batches of LMWH, each
20 calibrated for by similar antithrombotic effect
(factor X assay) have different optimal doses for
inhibition of TNF-α secretion. Moreover, there are
preparations of LMWH, such as Clexane® batch 4096,
which have no inhibitory effect on TNF-α, secretion at
25 any of the doses tried. Therefore, it may be
concluded that the antithrombotic effect of a LMWH
preparation is not related to the potential of the
LMWH preparation for inhibition of TNF-α secretion.
The two different bioassays are mediated by different
30 factors present in the preparations.

2. The ability of a particular dose of
LMWH to inhibit TNF-α secretion is positively
correlated with its ability to inhibit DTH reaction,
and the dose of a LMWH preparation optimally effective
35 in inhibiting TNF-α secretion is also optimally
effective in inhibiting the DTH reaction.

Table 5. Treatment of mice with different LMWHs inhibits both ex vivo TNF-α secretion and DTH responses.

Weekly treatment of mice with LMWH	Conc: μg/mouse	Ex vivo Con A induced TNF-α secretion		DTH responses	
		pg/ml	% Inhibition	DTH	% Inhibition
Fragmin®					
Batch 38609	None	450	—	25	—
	0.5	425	5	21	12
	1	400	12	23	10
	5	68	85	6	73
	10	350	22	20	20
	50	435	8	26	0
Batch 45389	None	320	—	28	—
	0.1	280	13	26	6
	1	70	78	4	89
	5	260	18	24	13
	10	290	10	26	6
	50	310	4	29	0
Clexane®					
Batch 2088	None	400	—	22	—
	0.1	360	10	17	23
	1	64	84	3	87
	5	152	38	13	41
	10	380	4	23	0
Batch 2066	None	350	—	23	—
	0.1	338	6	20	13
	1	185	54	8	65
	5	192	57	7	70
	10	186	55	7	70
Batch 4096	None	320	—	24	0
	1	335	0	27	0
	5	325	0	26	0
	10	330	0	24	0

5.7. Example 7: Treatment of Adjuvant Arthritis (AA) in Rats with Low Doses of LMWHs

Adjuvant arthritis is an experimental disease inducible in some strains of rats by immunizing them to antigens of Mycobacterium tuberculosis (Pearson, C.M., Proc. Soc. Exp. Biol. Med. (1956) 91:91). This experimental disease is considered to be a model of human rheumatoid arthritis

- 23 -

(Pearson, C.M., Arthritis Rheum. (1964) 7:80). The
arthritis appears to be caused by T lymphocytes that
recognize an antigen of M. tuberculosis that is
cross-reactive with structures in the joint tissues
5   (Cohen, I.R., et al., Arthritis Rheum. (1985) 28:841).
Lewis rats were immunized with M.
tuberculosis (1 mg) in oil to induce adjuvant
arthritis (Pearson, C.M., Proc. Soc. Exp. Biol. Med.
(1956) 91:91). Five days later the rats were
10   inoculated subcutaneously as indicated with the doses
of LMWH and/or heparin and scored for the development
of arthritis on a scale of 0-16 as described
(Holoshitz, Y., et al., Science (1983) 219:56). All
the experiments were performed with Fragmin®, Batch
15   38609.
In order to study the dose response to
Fragmin® (Fig. 1) rats immunized to induce AA were
injected subcutaneously weekly, starting on the 5th
day after injection with 0.5 μg (o), 1 μg (♦),
20   2 μg (●), 10 μg (◊), 15 μg (Δ) 20 μg (■); 30 μg (▲),
40 μg (x) and PBS control (□). The dose was maximally
effective in inhibiting arthritis.
The effect of the 20 μg dose of Fragmin® on
the course of AA is shown in Fig. 2: PBS control (□);
25   single treatment on 5th day (▲); daily (●); every 5th
day (o); weekly (■). It is shown that Fragmin
administration both at 5 day intervals and at 7 day
intervals inhibits arthritis.
Fig. 3 shows the effect of weekly
30   administration of Fragmin® (batch 38609) as compared
to standard heparin on AA. Lewis rats were immunized
to induce AA. Beginning on day 5, the rats were
inoculated subcutaneously at weekly intervals with a
20 μg dose of Fragmin® (●), heparin (o) or phosphate
35   buffered saline (PBS) control (□). The results show a
dramatic difference in potency between Fragmin® and

24

heparin: Fragmin® completely inhibited arthritis,
while heparin had no inhibitory effect.

No inhibitory effect on AA was found with
daily administration of a 20 μg dose of LMWH, although
5 surprisingly the inhibitory effect of heparin was
stronger than that of Fragmin® in daily
administration, as shown in (Fig. 4: Fragmin® (batch
38609)(•), heparin (o), PBS control (□)).

A similar inhibitory effect was observed
10 with several other LMWHs administered to Lewis rats
immunized to induce AA. Fig. 5 shows the results of
the injection of a 20 μg dose of Fraxiparin® (daily
(□); weekly (■)); Fraxiparine® (daily (Δ); weekly (▲)),
Lovenox®/Clexane® (daily (•); weekly (o)), and PBS
15 control (x). All the three LMWHs of different types
and sources showed a marked inhibition of arthritis,
when administered weekly, but not daily.

      5.8. Example 8: Treatment With LMWH Prevents
            Rejection of Allografts
20
            Wistar rats were subjected to allogeneic BN
heart transplant (Ono, K. and Linsay, E.S., J. Thorac.
Cardiovasc. Surg. (1969) 45:225-229). From the day
before transplantation, the rats were injected
subcutaneously at 7 day intervals with 20 μg of
25 Fragmin® or PBS control (Fig. 6, ■ and □, respectively)
and scored for survival. The day of rejection was
determined as the day the transplanted heart stopped
beating, assayed by palpation of the abdomen. Fig. 6
shows that the rats treated with the weekly dose of
30 LMWH had a markedly increased survival of the heart
allografts.

      5.9. Example 9: Biological Effect of LMWH
            On Insulin Dependent Diabetes
35   Mellitus (IDDM) of NOD Mice
            Mice of the NOD strain spontaneously develop
a form of type I insulin dependent diabetes mellitus

- 25 -

(IDDM) that is the accepted model for human IDDM
(Castano, L. and Eisenbarth, G.S., Annu. Rev. Immunol.
(1990) 8:647-679). The disease begins at 4-5 weeks of
age by the appearance of inflammation of the
5   pancreatic islets, insulitis. The insulitis
progressively damages the insulin-producing beta cells
which are sensitive to damage by TNF-α. At about 4-5
months of age, a sufficient number of beta cells are
destroyed so that diabetes becomes overt.

10   To test whether treatment with LMWH could
affect the IDDM process, a group of 10 female NOD mice
was treated with weekly subcutaneous injections of 5
μg per mouse of Fragmin® (batch 38609), the dose
determined to represent 12 mouse units per kg. A

15   group of 10 control mice were treated with injections
of saline. At the age of 5 months all the mice were
bled to determine the development of IDDM using a
standard procedure (Elias, D. et al., Proc. Natl.
Acad. Sci. U.S.A. (1990) 87:1576-1580). Fig. 7 shows

20   that the control mice ("none") had abnormal blood
glucose (400 mg/dl). In contrast the mice treated
with LMWH had a normal blood glucose (100 mg/dl).
Thus treatment with LMWH can indeed cure the IDDM
process.

25

      5.10.   Example 10: LMWH Treatment of Human DTH
              Figure 8 shows an experiment in which a 40
year old male volunteer weighing 85 kg was tested for
DTH reactivity to tetanus antigen (Merieux skin test

30   applicator). About 18 mm of induration was measured
at 24 and 48 hours. The volunteer was then treated
with a subcutaneous injection of Fragmin® (batch
38609) 3 mg. Five days later the volunteer was again
tested for his DTH response to tetanus and the

35   induration was inhibited to about 5 mm. The volunteer
was tested again for DTH 3 weeks later ("Recovery")
and the test showed positive reactivity (23 mm of

induration at 24 and 48 hours). The volunteer was
then treated with Fragmin® as before and the DTH
reactivity was measured again 7 days later ("7 days
post"). Again the DTH was inhibited to about 5 mm of
5   induration. Recovery of DTH again was found 3 weeks
later. Thus, LMWH at a dose of less than 5 mg can
inhibit DTH in humans at treatment intervals of 5 and
7 days.
        It should be apparent to those skilled in
10   the art that other compositions and methods not
specifically disclosed in the instant specification
are, nevertheless, contemplated thereby. Such other
compositions and methods are considered to be within
the scope and spirit of the present invention. Hence,
15   the invention should not be limited by the description
of the specific embodiments disclosed herein but only
by the following claims.

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- 27 -

WHAT IS CLAIMED IS:

1.   A pharmaceutical composition for the
prevention and/or treatment of pathological processes
5   involving the induction of TNF-α secretion comprising
a pharmaceutically acceptable carrier and a low
molecular weight heparin (LMWH) present in a low
effective dose for administration at intervals of
about 5-8 days and which LMWH is capable of inhibiting
10   in vitro TNF-α secretion by resting T cells and/or
macrophages in response to T cell-specific antigens,
mitogens, macrophage activators, disrupted
extracellular matrix (dECM), laminin, fibronectin, or
other ECM components.
15

2.   The pharmaceutical composition of claim
1 in which the LMWH has an average molecular weight of
from about 3,000 to about 6,000.

20   3.   The pharmaceutical composition of claim
1 or 2 for administration every fifth day.

4.   The pharmaceutical composition of claim
1 or 2 for administration every seventh day.
25

5.   The pharmaceutical composition of claim
1, 2, 3, or 4 in which said LMWH is further
characterized as being capable of inhibiting
experimental delayed type hypersensitivity (DTH)
30   reactions.

6.   The pharmaceutical composition of claim
1, 2, or 5 for subcutaneous or intravenous
administration.

35

2 ?

7.   The pharmaceutical composition of claim
1 or 2 which is effective to delay or prevent
allograft rejection.

5   8.   The pharmaceutical composition of claim
1 or 2 in which said pathological processes include
autoimmune diseases, allergy, inflammatory diseases,
and other pathological processes related thereto.

10   9.   The pharmaceutical composition of claim
1 or 2 in which said pathological processes are
related to AIDS.

                   10.   The pharmaceutical composition of claim
15 1 or 2 for the treatment of diabetes type I,
periodontal disease, inflammatory bowel disease,
uveitis, rheumatic diseases or multiple sclerosis.

                   11.   The pharmaceutical composition of claim
20 9 for the treatment of rheumatoid arthritis.

                   12.   The pharmaceutical composition of claim
7 for the treatment of chronic inflammation.

25   13.   The pharmaceutical composition of claim
7 for the prevention and/or treatment of
atherosclerosis, vasculitis, and pathological
processes related thereto.

30   14.   The pharmaceutical composition of claim
2 in which said LMWH is present in a single low dose
unit of less than 5 mg.

                   15.   The pharmaceutical composition of claim
35 13 in which said LMWH is present in a single low
dosage unit of less than 3 mg.

29

16. The pharmaceutical composition of claim
14 in which said LMWH is present in a single low dose
unit of from about 0.3 to about 3 mg.

5   17. The pharmaceutical composition of claim
14 in which said LMWH is present in a single low dose
unit of from 1 to 1.5 mg.

         18. A method of using a low molecular
10 weight heparin (LMWH) which is capable of inhibiting
in vitro TNF-α secretion by resting T cells and/or
macrophages in response to T cell-specific antigens,
mitogens, macrophage activators, disrupted
extracellular matrix (dECM), laminin, fibronectin, or
15 other ECM components for the preparation of a
pharmaceutical composition to be administered at
intervals of about 5-8 days for the prevention and/or
treatment of pathological processes involving
induction of TNF-α secretion which comprises combining
20 a low effective dose of said LMWH with a
pharmaceutically acceptable carrier.

         19. The method of claim 17 in which said
pharmaceutical composition is to be administered every
25 seventh day.

         20. The method of claim 17 in which said
LMWH has an average molecular weight of from about
3,000 to about 6,000.
30
         21. The method of claim 17 in which said
low effective dose is less than 5 mg of said LMWH.

         22. The method of claim 17 in which said
35 pathological processes include allograft rejection,
allergy, autoimmune and inflammatory diseases.

23. The method of claim 17 in which said
pathological processes are related to AIDS.

24. The method of claim 17 in which said
5 pathological processes is related to diabetes type I,
periodontal disease, inflammatory bowel disease,
uveitis, rheumatic diseases, chronic inflammation,
multiple sclerosis, atherosclerosis, and vasculitis.

10   25. A pharmaceutical composition to be
administered at intervals of about 5-8 days for the
prevention and/or treatment of pathological processes
involving the induction of TNF-α secretion comprising
a pharmaceutically acceptable carrier and a low
15 molecular weight heparin (LMWH) present in a low
effective dose and which LMWH is capable of inhibiting
experimental delayed type hypersensitivity (DTH)
reactions to an applied antigen as evidenced by a
reduction in the induration observed after the
20 application of said antigen to the skin five to seven
days after the administration of said pharmaceutical
composition relative to the induration observed after
the application of said antigen to the skin in the
absence of or after recovery from the administration
25 of said pharmaceutical composition.

26. The pharmaceutical composition of claim
24 in which said LMWH has an average molecular weight
of from about 3,000 to about 6,000.
30
27. The pharmaceutical composition of claim
24 in which said antigen is selected from the group
consisting of tetanus, myelin basic protein, purified
protein derivative, and oxazolone.
35
28. A method of treating a subject
suffering from a pathological process involving

induction of TNF-α secretion comprising administering
to such subject a pharmaceutical composition of claim
1, 2, 24, or 25 at intervals of about 5-8 days.

5   29.   The method of claim 27 in which said
interval between administrations is five days.

           30.   The method of claim 27 in which said
interval between administrations is seven days.

10

           31.   The method of claim 27 in which said
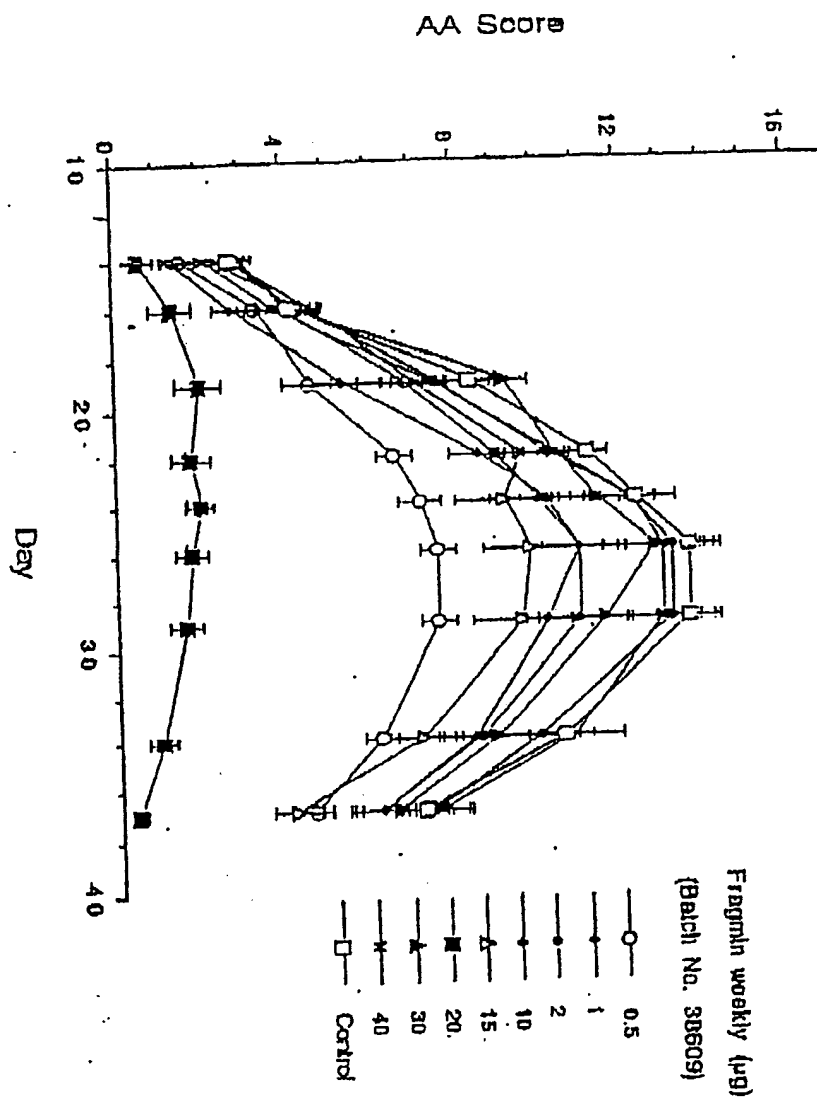pharmaceutical composition contains less than about 5
mg of said LMWH.

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1/8

2/8

Figure 2

The effect of fragmin (batch 3B609) on the course of AA

Legend:
- □ 20 μg dose P.B.S.
- ▲ single treatment
- ● daily
- ○ every 5 days
- ■ weekly

3/8
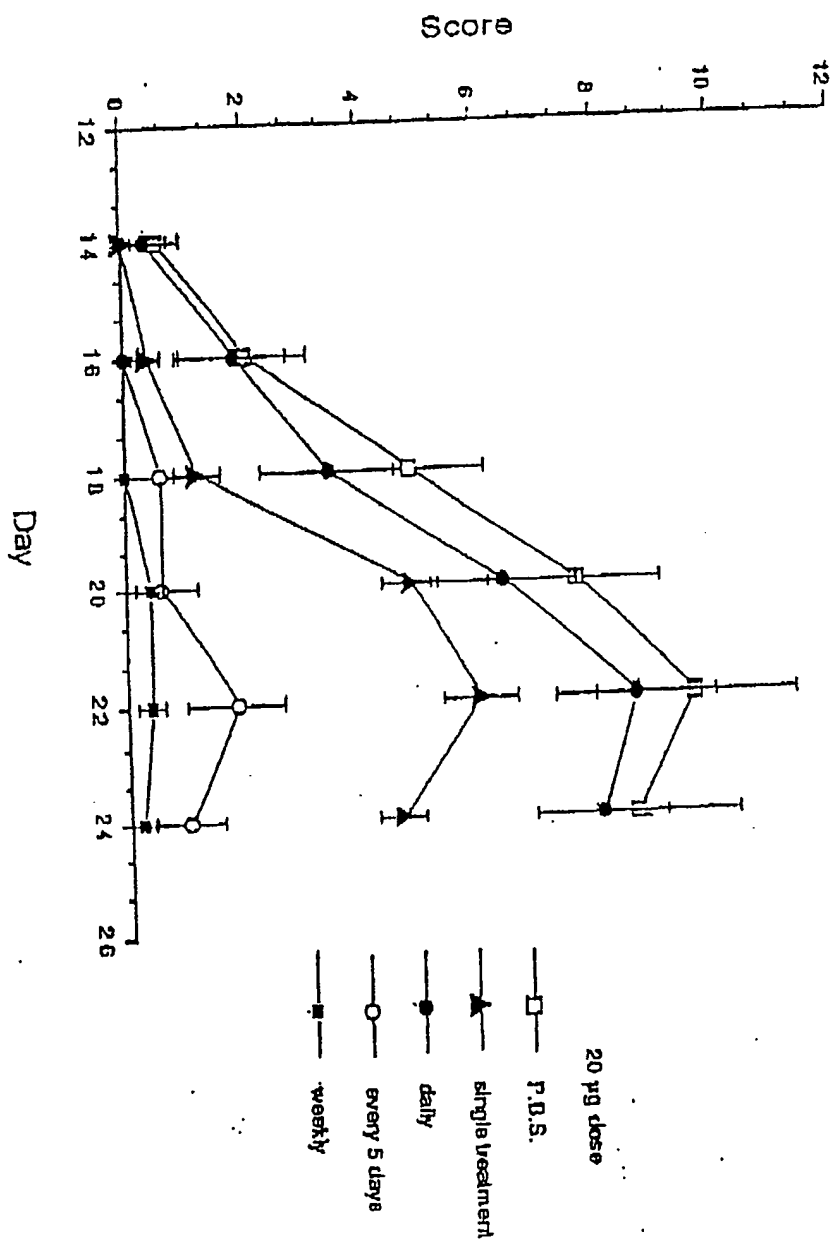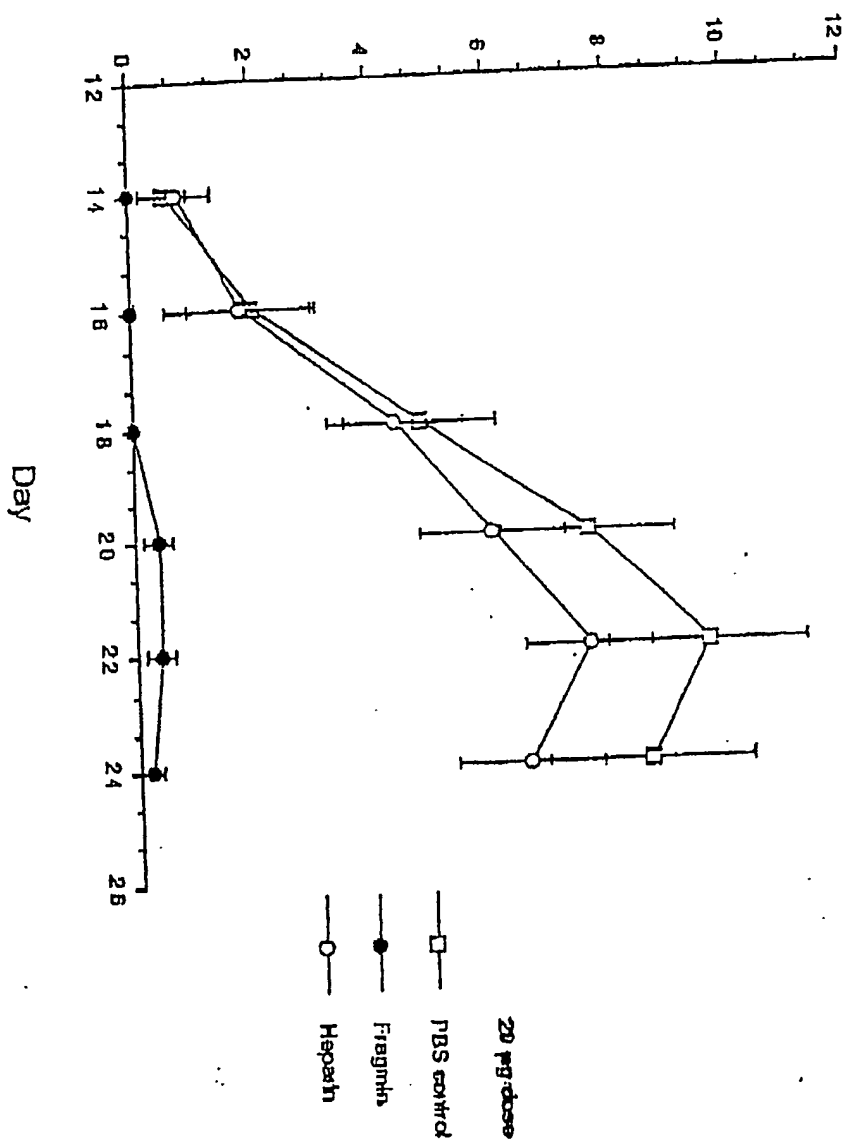
Figure 3.

The effect of weekly Fragmin/Heparin on AA
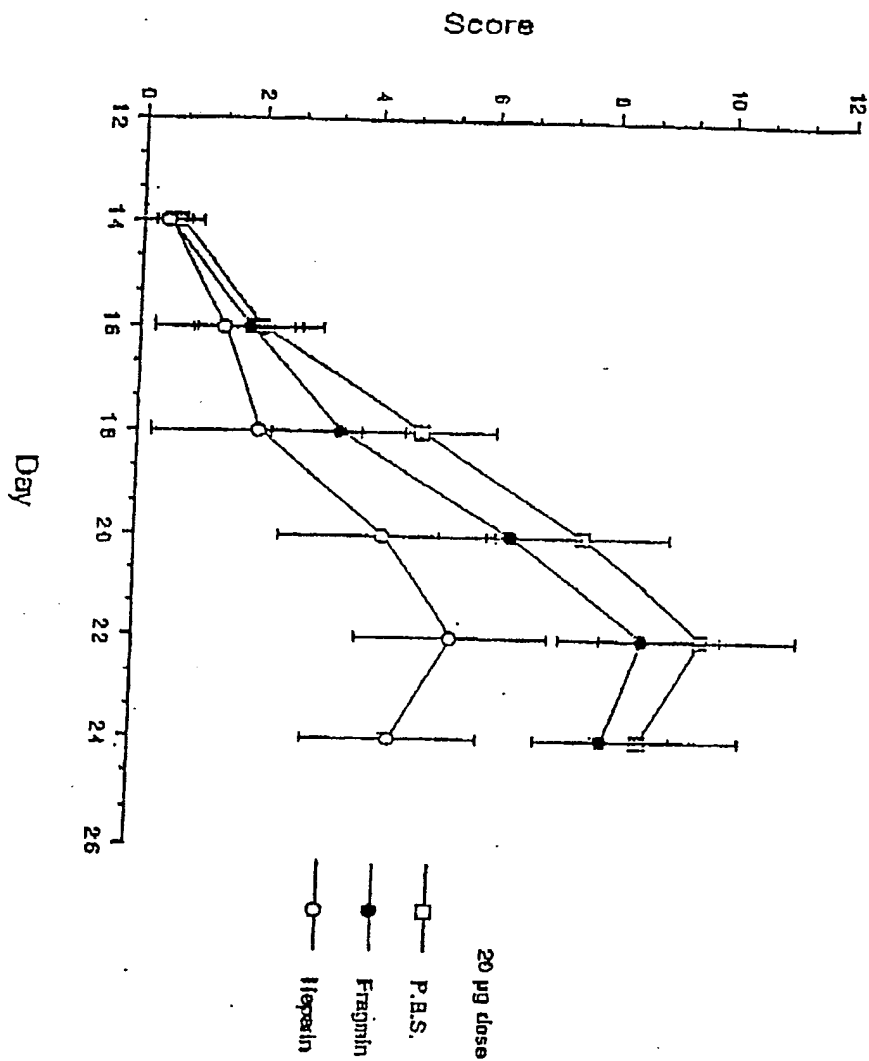
4/8

Figure 4

The effect of Fragmin\Heparin daily on AA

5/8

Figure 5

6/8

Figure 6

7/8
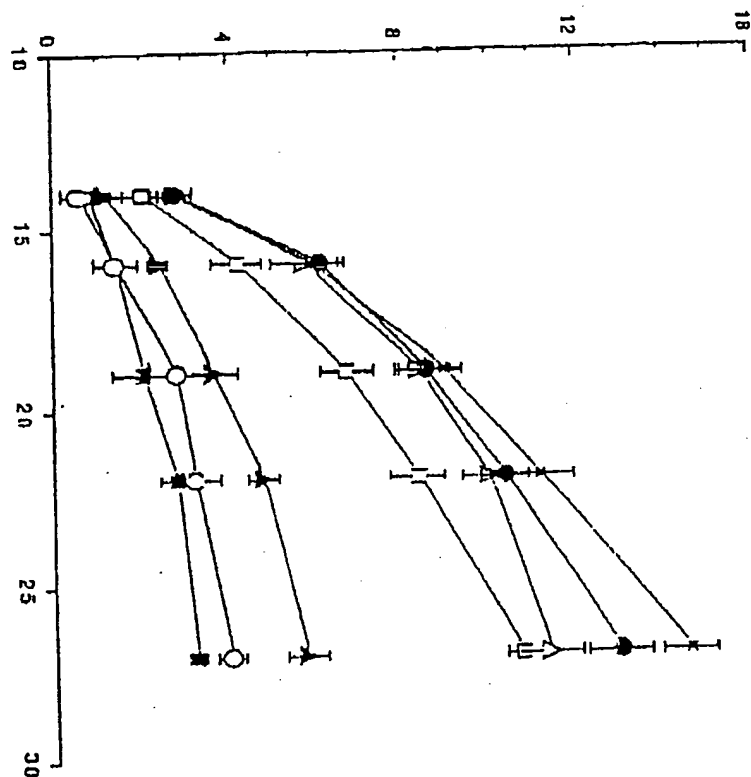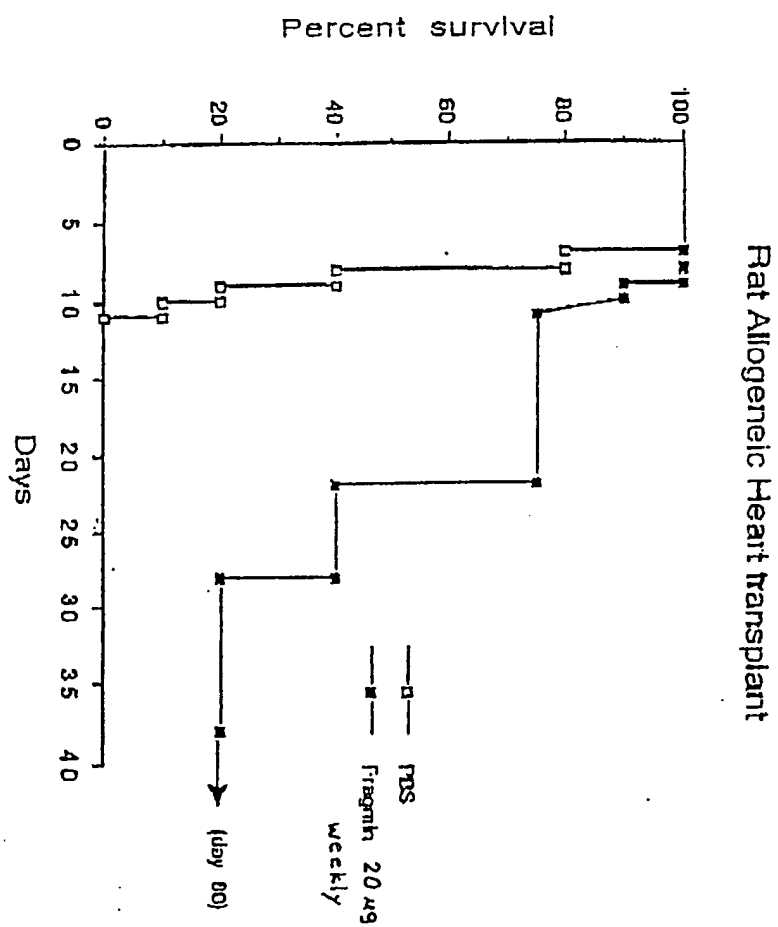
Fragmin Treatment Aborts Spontaneous IDDM in NOD Mice

Blood Glucose (mg/dl)
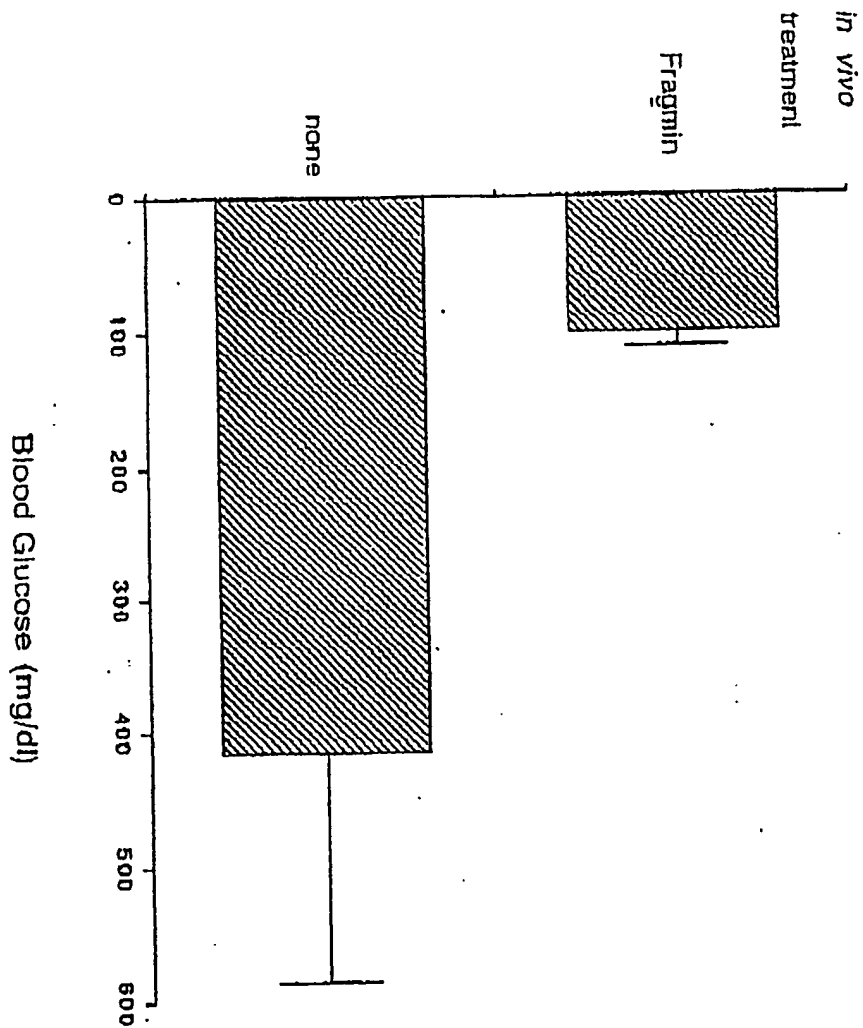
Figure 7

8/8
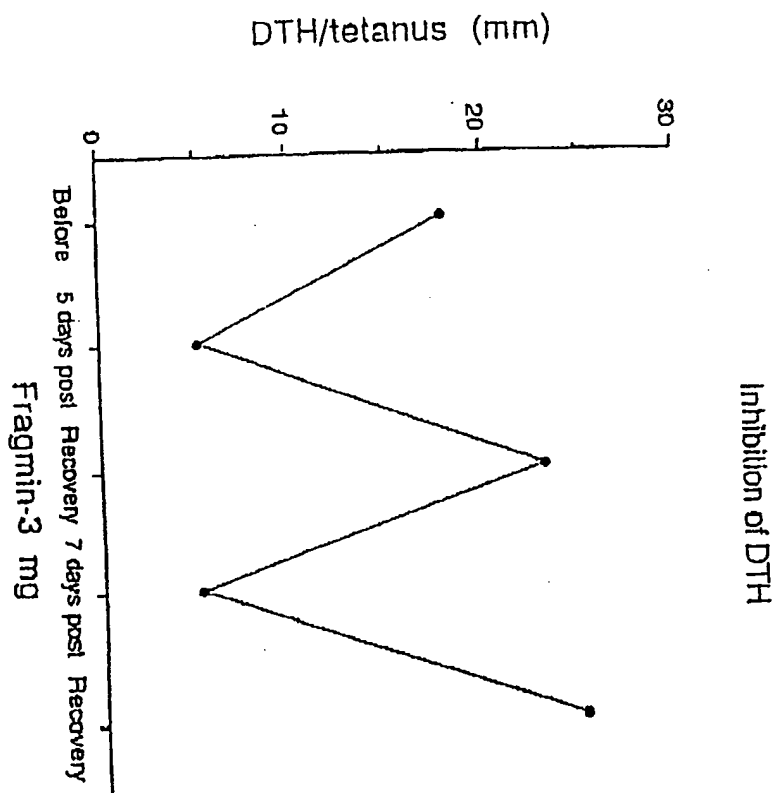
Figure 8

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 31/725
US CL :514/54, 56; 536/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/54, 56; 536/21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CELLULAR IMMUNOLOGY Vol. 82, issued 1983, M.S.Sy et al, "Inhibition of Delayed-Type Hypersensitivity by Heparin Depleted of Anticoagulant Activity", pages 23-32, 24-29.	1-31
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 263, no. 26, issued 15 September 1988, R.J. Linhardt et al, "Homogeneous, Structurally Defined Heparin-Oligosaccharides with Low Anticoagulant Activity Inhibit the Generation of the Amplification Pathway C3 Convertase in Vitro", pages 13090-13096, see entire document.	1-31.
Y	PHYSIOLOGY, CHEMISTRY AND PHYSICS, Vol. 10, issued 1978, I. Kiricuta et al, "Prophylaxis of Metastases Formation by Prolonged Non-specific Immunostimulation Associated with Heparin Therapy", pages 247-253, see entire document.	1-31
Y	ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, Vol. 463, (Colloq. Biol. Sci. 2nd), issued 1986, I.D. Goldberg et al, "The in vivo Effects of Heparin Fractions on the Development of Lung Metastasis in Murine Fibrosarcoma", pages 289-291, see entire document.	1-31

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	
"A"	document defining the general state of the art which is not considered to be part of particular relevance	
"E"	earlier document published on or after the international filing date	
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior to the international filing date but later than the priority date claimed	

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 JULY 1992	1 7 JUL 1992

Name and mailing address of the ISA/	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	NANCY S. CARSON
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Form PCT/ISA/210 (second sheet)(July 1992)*

International application No.

PCT/US92/03626

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AMERICAN JOURNAL OF PATHOLOGY, Vol. 82, no. 1, Issued January 1976, R. W. Colman et al, "Hyperacute Renal Allograft Rejection in the Primate", pages 25-42, see entire document.	1-31
Y	THROMBOSIS RESEARCH, Vol. 42, issued 1986, J. Westwick et al, "Comparison of the Effects of Low Molecular Weight Heparin and Unfractioned Heparin on Activation of Human Platelets in vitro", page 435-447, see entire document.	1-31
Y	US, A, 4,788,307 (Lormeau et al) 29 November 1988, cols. 2-4.	1-31
Y	US, A, 4,281,108 (Fussi) 28 July 1981, cols. 3-5.	1-31